(2019). Inherited missense variants that affect GFI1B function do not necessarily cause bleeding diatheses. *Haematologica*, *104*(6), e260-e264. [207712]. https://doi.org/10.3324/haematol.2018.207712

Inherited missense variants that affect GFI1B function are not necessarily causal to bleeding diatheses.

Rinske van Oorschot*,[1] Anna E. Marneth*,[1] Saskia M. Bergevoet,[1] Maaike G.J.M. van Bergen,[1] Kathelijne Peerlinck,[2] Claire E. Lentaigne,[3] Carolyn M. Millar,[3,4] Sarah K. Westbury,[5] Remi Favier,[6] Wendy N. Erber,[7] Ernest Turro,[8-11] Joop H. Jansen,[1] Willem H. Ouwehand,[8-10, 12-14] Harriet L. McKinney,[8-10] NIHR BioResource (collaborative group),[10] Kate Downes,[8-10] Kathleen Freson,[2,10] and Bert A. van der Reijden[1]

[1]Department of Laboratory Medicine, Laboratory of Hematology, Radboudumc, Radboud Institute for Molecular Life Sciences (RIMLS), Nijmegen, The Netherlands
[2]Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium
[3]Centre for Haematology, Hammersmith Campus, Imperial College Academic Health Sciences Centre, Imperial College London, London, United Kingdom
[4]Imperial College Healthcare NHS Trust, London, United Kingdom
[5]School of Cellular and Molecular Medicine, University of Bristol, Bristol, United Kingdom
[6]Service d'Hematologie Biologique, Assistance-Publique Hôpitaux de Paris, Centre de Référence des Pathologies Plaquettaires, Hôpital Armand Trousseau, Paris, France.
[7]School of Biomedical Sciences, University of Western Australia, Crawley, Western Australia, Australia; PathWest Laboratory Medicine, Nedlands, Western Australia, Australia
[8]Department of Haematology, University of Cambridge, Cambridge Biomedical Campus, Cambridge, United Kingdom
[9]National Health Service Blood and Transplant (NHSBT), Cambridge Biomedical Campus, Cambridge United Kingdom
[10]NIHR BioResource, Cambridge University Hospitals, Cambridge Biomedical Campus, Cambridge, United Kingdom
[11]Medical Research Council Biostatistics Unit, University of Cambridge, Forvie Site, Cambridge Biomedical Campus, Cambridge, United Kingdom
[12]Department of Human Genetics, The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom
[13]Strangeways Research Laboratory, The National Institute for Health Research (NIHR) Blood and Transplant Unit in Donor Health and Genomics at the University of Cambridge, University of Cambridge, Cambridge, United Kingdom
[14]BHF Centre of Excellence, Division of Cardiovascular Medicine, Addenbrooke's Hospital, Cambridge Biomedical Campus, Cambridge, United Kingdom

* R.O. and A.E.M. contributed equally to this work.
Running head: Characterization of inherited *GFI1B* variants
Corresponding author: Bert A. van der Reijden, Bert.vanderReijden@radboudumc.nl

Main text word count: 1489
Number of Figures/Tables: 3
Number of supplemental files: 1

Acknowledgements

This work was supported by grants from the Radboudumc (R.O.), the Landsteiner Foundation for Blood Transfusion Research (project 1531) (M.G.J.M.B.), and the National Institute for Health Research (NIHR, grant number RG65966). A.E.M. is supported by EMBO long-term fellowship grant ATLF 268-2017. K.D. is a HSST trainee supported by Health Education England. K.F. and K.P. are holders of the SOBI chair and are supported by the Research Council of the University of Leuven (BOF KU Leuven, Belgium, OT/14/098). C.M.M. is supported by the NIHR Imperial College Biomedical Research Centre.

Several types of *GFI1B* variants have been identified in patients with inherited bleeding and platelet disorders. This includes dominant-negative truncating variants affecting DNA binding,[1-4] missense variants of which the molecular mechanism is unclear,[5-7] and variants changing the amount and ratio of GFI1B isoforms (Figure S1).[7, 8] The severity of the bleeding disorder may differ depending on the type of variant, but frequent abnormalities include macrothrombocytopenia, a reduction in α-granule numbers, and platelet CD34 expression. In this study we performed a molecular and/or clinicopathological characterization of eight GFI1B variants in non-DNA binding domains (Figure S1). These variants were previously identified by the NIHR BioResource rare disease study in cases with an assumed inherited bleeding or platelet disorder.[9] Molecular characterization was not performed for D23N, since the minor allele frequency in the gnomAD database deemed too high for a causal variant. From the characterization of the other variants we can conclude that although some have a clear effect on GFI1B function, they are not necessarily sufficient to cause bleedings on their own.

Previously, we used the megakaryoblast cell line MEG-01 to study the effect of GFI1B and the proven pathogenic GFI1B-Q287* variant on cell expansion. In expansion-competition cultures containing transduced and non-transduced cells, MEG-01 cells ectopically expressing GFI1B were overgrown by non-transduced cells, while the opposite was observed following expression of GFI1B-Q287* (Figure 1; manuscript resubmitted September 2018). Thus, forced GFI1B expression inhibits MEG-01 cell expansion whereas dominant-negative GFI1B-Q287* results in enhanced expansion. The latter is in line with elevated megakaryocyte numbers observed in a bone marrow specimen of a GFI1B p.Q287* affected individual.[1] To investigate the (functional) effect of GFI1B variants, we retrovirally expressed them in MEG-01 cells and performed the expansion-competition culture described above. GFI1B and GFI1B-Q287* were taken along as references. Two variants, one in the intermediate domain (G139S) and one in zinc finger (znf) 2 (G198S), did not affect the inhibitory function of wild type GFI1B on MEG-01 proliferation (Figure 1A, Figure 1B). The R190W variant, located between znf1 and znf2, rendered the protein less effective at inhibiting MEG-01 proliferation (Figure 1C), whilst both the znf1 variant C168F and the truncated variant Q89fs rendered the protein completely inactive (Figure 1D, Figure 1E). Interestingly, expression of znf1 H181Y and R184P variants resulted in increased MEG-01 cell proliferation, although to a lesser extent than cells expressing GFI1B-Q287* (Figure 1F, Figure 1G). To further study H181Y and R184P, we introduced these variants separately in GFI1B-Q287*. This led to partial inhibition of the growth stimulating effect of GFI1B-Q287* (Figure 2A, Figure 2B), indicating that amino acids H181 and R184 are important for the effect of GFI1B-Q287* on MEG-01 proliferation. These findings clearly demonstrate that different variants have qualitatively distinct effects on the function of GFI1B, and that znf1 is important in regulating MEG-01 proliferation.

The increased MEG-01 expansion caused by GFI1B-H181Y and GFI1B-R184P suggests that these variants, like GFI1B-Q287*, act in a dominant-negative manner. However, the molecular mechanism might be different, because these variants are not located in the DNA binding domain like GFI1B-Q287*. GFI1B is a repressive transcription factor that inhibits its own transcription and that of its paralogue *GFI1*.[10, 11] GFI1B-Q287* has lost this repressive function.[1] To study if the variants affect the repressive function of GFI1B, we performed gene reporter assays using the *Gfi1* promoter. Remarkably, all tested GFI1B missense variants, including GFI1B-H181Y and GFI1B-R184P, repressed the *Gfi1* promoter to a similar extent as wild type GFI1B (Figure 2C). However, results obtained in transient gene repression assays may not reflect effects on endogenous target genes. We therefore analyzed the effects of GFI1B-H181Y and GFI1B-R184P on endogenous *GFI1B* expression. Wild type GFI1B, GFI1B-Q287*, GFI1B-H181Y, and GFI1B-R184P were expressed in MEG-01 cells, followed by

endogenous *GFI1B* mRNA expression analysis. In line with earlier reports, wild type GFI1B inhibited endogenous *GFI1B* expression.[12] In contrast, GFI1B-Q287*, as well as GFI1B-H181Y and GFI1B-R184P did not repress endogenous *GFI1B* expression to the same extent as wild type GFI1B (Figure 2D). This indicates that not only the DNA binding znfs, but also amino acids H181 and R184 are required for efficient repression of endogenous *GFI1B*.

The LSD1-RCOR1-HDAC co-repressor complex is one of the main epigenetic regulatory complexes recruited by GFI1B to induce transcriptional repression. To study whether GFI1B-H181Y- and GFI1B-R184P-induced MEG-01 expansion depends on an interaction with this complex, we co-introduced a P2A mutation in the GFI1B-H181Y or GFI1B-R184P variants. The P2A mutation in the N-terminal SNAG domain of GFI1B abrogates its interaction with LSD1,[13] and nullifies the inhibitory effect of wild type GFI1B and stimulatory effect of GFI1B-Q287* on MEG-01 proliferation (manuscript resubmitted September 2018). Expression of the P2A-H181Y and P2A-R184P double mutants resulted in expansion rates similar to empty vector transduced cells (Figure 2E). This strongly suggests that H181Y and R184P variants require the LSD1 interaction to exert their effect on MEG-01 expansion.

The functional data were subsequently correlated with clinical and laboratory features of patient samples to improve classification of the *GFI1B* variants according to ACMG guidelines[14] (Table S1). A minimal set of genetic, clinical and laboratory features have already been published in Chen *et al*., supplementary table ST15.[9] For this study, we expanded clinical and laboratory phenotype studies for the H181Y and R184P variants, because these GFI1B variants had similar functional effects in the MEG-01 cell models as the proven pathogenic GFI1B-Q287* variant. In addition, we performed clinical and laboratory phenotype studies for R190W variant carriers.

The variants G139S and G198S were classified as 'Benign' as they showed similar inhibition of MEG-01 expansion as wild type GFI1B, and have a relatively high minor allele frequency in gnomAD. Further, the thrombocytopenia in patient P9 with G198S was explained by a pathogenic ACTN1 variant (p.R46Q)[15]. Variants R190W, C168F and Q89fs did not inhibit MEG-01 expansion to the same extent as wild type GFI1B (loss of function effect). R190W platelets were weakly CD34-positive, but R190W in patients P8.1 and P8.2 did not co-segregate with bleeding or result in abnormal α-granules (Table S1; Figures S3-5). Moreover, patient P7 with the same R190W variant was explained by a pathogenic variant in WAS (p.R364*), resulting in a 'Benign' classification for R190W. Patient P4 with a homozygous C168F variant suffered from clinical bleeding symptoms with thrombocytopenia and platelet aggregation dysfunction. Unlike P4, heterozygous C168F patients studied by Rabbolini and colleagues only displayed macrothrombocytopenia with platelet CD34 expression (partial effect on the phenotype).[7] C168F is predicted to disrupt znf1 structure and thereby GFI1B function.[9] This was confirmed in functional experiments performed here (Figure 1D) and by Rabbolini *et al*. showing that C168F disrupts the repressive function of GFI1B gene expression.[7] C168F was classified as a 'variant of unknown significance' (VUS); further studies in the affected patient or of family members was not possible. A 90-year old woman (deceased) carrying the Q89fs variant and without affected siblings had mild thrombocytopenia with bleeding, platelet dysfunction and significantly reduced α-granule numbers; a phenotype very similar to previously described *GFI1B* pathogenic variants (Table S1; Figure S5).[1, 2, 4] The Q89fs variant does not repress the *Gfi1* promoter to the same degree as wild type GFI1B and the missense variants. However, it must be noted that we could only detect the truncated protein after proteasome inhibition, suggesting it is instable (Figure S6). If this is also the case in patient cells, the Q89fs variant would lead to haploinsufficiency. This variant was classified as VUS.

The R184P and H181Y variants stimulated MEG-01 proliferation and failed to repress endogenous *GFI1B* expression in a similar way as the pathogenic Q287* variant. These missense variants were absent from the gnomAD database and co-segregation studies were performed (Figure 3). Both the propositus (P6.1) and her father (P6.2), who are carriers of R184P, showed a small number of hypogranular platelets and platelet CD34 expression (Table S1; Figures S3-4). P6.1 had a normal platelet count whereas her father (P6.2) had mild thrombocytopenia. Importantly, neither parent had clinical bleeding symptoms or platelet dysfunction (Table S1; Figure 3A). Following ACMG criteria, the R184P variant was classified as VUS. For the propositus (P5.1) with the H181Y variant, three affected relatives (P5.2, P5.4-5) and one non-affected (P5.3) relative were screened and the variant co-segregated with clinical bleeding symptoms, platelet dysfunction and CD34-positive platelets (Table S1; Figure S3; Figure 3B). Affected individuals P5.1 and P5.2 had normal platelet counts with few large platelets and a significant reduction of α-granules (Table S1; Figures S4-5). The functional and segregation data suggest that the H181Y variant is causal of bleeding and platelet dysfunction but does not result in thrombocytopenia. Following ACMG guidelines, H181Y was classified as a VUS (Table S1).

We conclude Q89fs, C168F, H181Y, and R184P affect GFI1B function, but are not necessarily sufficient to cause bleedings on their own. Still, their identification and documentation, even when classified as VUS, will help to distinguish pathological from non-pathological *GFI1B* variants and increase our understanding of GFI1B functional domains. The identification of additional patients with similar variants will be essential to clarify their exact role for platelet phenotypes and bleeding.

Authorship

Contribution: R.O., A.E.M., S.M.B., and M.G.J.M.B performed *in vitro* experiments; K.F., W.N.E., H.M. and K.D. performed EM, blood smears and CD34 expression measurements; Patients were followed by K.P., C.L., C.M.M., S.K.W., R.F., and W.H.O.; E.T. and W.H.O. analyzed and coordinated the genetic studies; R.O., A.E.M., and K.F. analyzed results and made the figures; R.O., A.E.M., B.A.R., J.H.J., and K.F. designed the research and wrote the paper; All authors have read and agreed to the contents of the paper.

Conflict of Interest Disclosure: The authors declare no competing financial interests.

Correspondence: Bert A. van der Reijden, Department of Laboratory Medicine, Laboratory of Hematology, Radboudumc, Geert Grooteplein zuid 8, 6525 GA Nijmegen, The Netherlands; e-mail: Bert.vanderReijden@radboudumc.nl

References

1. Monteferrario D, Bolar NA, Marneth AE, et al. A dominant-negative GFI1B mutation in the gray platelet syndrome. N Engl J Med. 2014;370(3):245-253.
2. Stevenson WS, Morel-Kopp MC, Chen Q, et al. GFI1B mutation causes a bleeding disorder with abnormal platelet function. J Thromb Haemost. 2013;11(11):2039-2047.
3. Marneth AE, van Heerde WL, Hebeda KM, et al. Platelet CD34 expression and alpha/delta-granule abnormalities in GFI1B- and RUNX1-related familial bleeding disorders. Blood. 2017;129(12):1733-1736.
4. Kitamura K, Okuno Y, Yoshida K, et al. Functional characterization of a novel GFI1B mutation causing congenital macrothrombocytopenia. J Thromb Haemost. 2016;14(7):1462-1469.
5. Uchiyama Y, Ogawa Y, Kunishima S, et al. A novel GFI1B mutation at the first zinc finger domain causes congenital macrothrombocytopenia. Br J Haematol. 2017;
6. Ferreira CR, Chen D, Abraham SM, et al. Combined alpha-delta platelet storage pool deficiency is associated with mutations in GFI1B. Mol Genet Metab. 2017;120(3):288-294.
7. Rabbolini DJ, Morel-Kopp MC, Chen Q, et al. Thrombocytopenia and CD34 expression is decoupled from alpha-granule deficiency with mutation of the first growth factor-independent 1B zinc finger. J Thromb Haemost. 2017;15(11):2245-2258.
8. Schulze H, Schlagenhauf A, Manukjan G, et al. Recessive grey platelet-like syndrome with unaffected erythropoiesis in the absence of the Splice Isoform GFI1B-p37. Haematologica. 2017;102(9):102(109):e375-e378.
9. Chen L, Kostadima M, Martens JH, et al. Transcriptional diversity during lineage commitment of human blood progenitors. Science. 2014;345(6204):1251033.
10. Anguita E, Villegas A, Iborra F, Hernandez A. GFI1B controls its own expression binding to multiple sites. Haematologica. 2010;95(1):36-46.
11. Vassen L, Fiolka K, Mahlmann S, Moroy T. Direct transcriptional repression of the genes encoding the zinc-finger proteins Gfi1b and Gfi1 by Gfi1b. Nucleic acids research. 2005;33(3):987-998.
12. Huang DY, Kuo YY, Chang ZF. GATA-1 mediates auto-regulation of Gfi-1B transcription in K562 cells. Nucleic acids research. 2005;33(16):5331-5342.
13. Saleque S, Kim J, Rooke HM, Orkin SH. Epigenetic regulation of hematopoietic differentiation by Gfi-1 and Gfi-1b is mediated by the cofactors CoREST and LSD1. Molecular cell. 2007;27(4):562-572.
14. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-424.
15. Westbury SK, Turro E, Greene D, et al. Human phenotype ontology annotation and cluster analysis to unravel genetic defects in 707 cases with unexplained bleeding and platelet disorders. Genome Med. 2015;7(1):36.

Figure legends

Figure 1. GFI1B variants have different effects on GFI1B function. Expansion competition cultures of MEG-01 cells transduced with flag-tagged GFI1B variants (A) G139S (B) G198S (C) R190W (D) C168F (E) Q89fs (F) H181Y (G) R184P. GFI1B-Q287*-flag, GFI1B-p37-flag wild type (WT), and empty vector (EV) were taken along as controls. Fold change of GFP% to GFP% at day 5 (first GFP measurement) is presented on the y-axis. Results show mean ± standard error of the mean, and two-tailed paired t-tests were performed on day 26 to determine statistical significance *P<0.05, **P <0.01. Of note, all MEG-01 transduced cells showed increased GFI1B mRNA expression indicating expression of the retroviral vector (Figure S2).

Figure 2. Functional effect of GFI1B variants H181Y and R184P. (A-B) Expansion competition cultures of MEG-01 cells transduced with flag-tagged GFI1B-H181Y+Q287* (A), or GFI1B-R184P-Q287* (B). Empty vector (EV) and GFI1B-Q287*-flag transduced cells taken along as reference. (C) Dual luciferase reporter assays in HEK293FT cells transfected with Renilla luciferase construct, *Gfi1* promoter Firefly luciferase construct, and empty vector (EV), wild type GFI1B-p37-flag (WT-p37), wild type GFI1B-p32-flag (WT-p32, lacking coding exon 4 and therefore amino acids 171-216 corresponding to zinc finger 1 and 2), or GFI1B-flag variants. Firefly/Renilla luciferase ratios are normalized to EV transfected cells. Results show mean ± standard deviation, and two-tailed paired t-tests were performed to determine statistical significance between WT-p37 and the other conditions. Corresponding Western blots showing expression of the flag-tagged GFI1B proteins and the GAPDH loading control are depicted below the graph. (D) 5'UTR *GFI1B* expression in GFP positive cells from MEG-01 expansion competition cultures, FACS-sorted 23 days after transduction. *GFI1B* expression is normalized to *GAPDH* expression. Results show mean ± standard deviation, and two-tailed paired t-tests were performed to determine statistical significance. (E) Expansion competition cultures of MEG-01 cells transduced with flag-tagged GFI1B-H181Y+P2A, or GFI1B-R184P-P2A. EV transduced cells taken along as reference. *P<0.05, **P <0.01, ***P <0.001

Figure 3. Pedigrees for families harboring GFI1B variants H181Y (A) and R184P (B). The propositus (arrow) and the family members with signs of pathological bleeding are indicated with a black filled symbol. GFI1B variant status, patient identifier, platelet count (PLT), platelet CD34 expression, and the ISTH bleeding assesment tool (BAT) score are indicated for each patient. Normal range for the ISTH BAT score is <4 in adult males, <6 in adult females and <3 in children. P5.4 has less haemostatic challenges than the other siblings. Additional clinical and laboratory data obtained in patient samples can be found in Table S1.

Figure 1

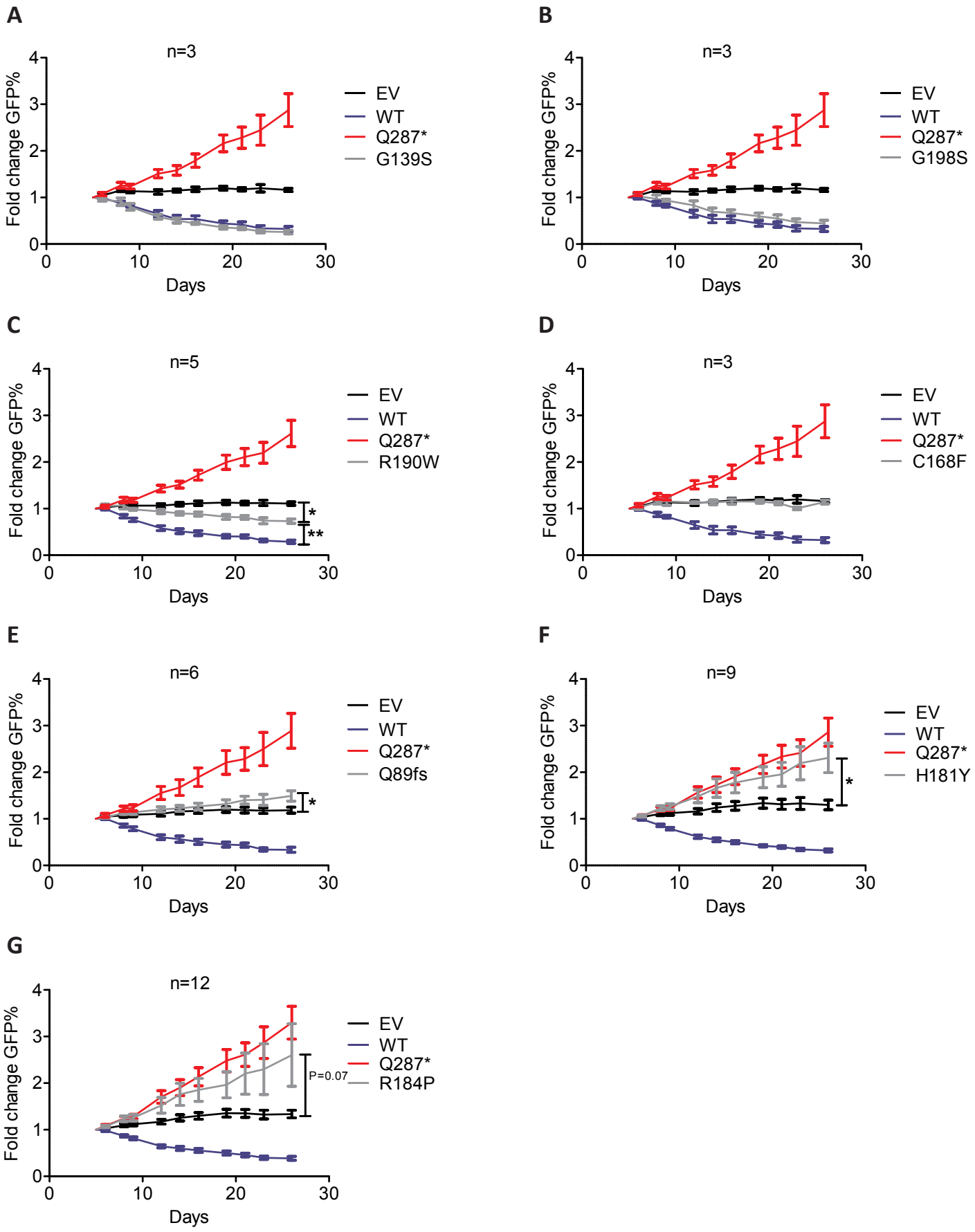

A
n=3
Fold change GFP%
Days
EV
WT
Q287*
G139S
B
n=3
Fold change GFP%
Days
EV
WT
Q287*
G198S
C
n=5
Fold change GFP%
Days
EV
WT
Q287*
R190W
*
**
D
n=3
Fold change GFP%
Days
EV
WT
Q287*
C168F
E
n=6
Fold change GFP%
Days
EV
WT
Q287*
Q89fs
*
F
n=9
Fold change GFP%
Days
EV
WT
Q287*
H181Y
*
G
n=12
Fold change GFP%
Days
EV
WT
Q287*
R184P
P=0.07

Figure 2

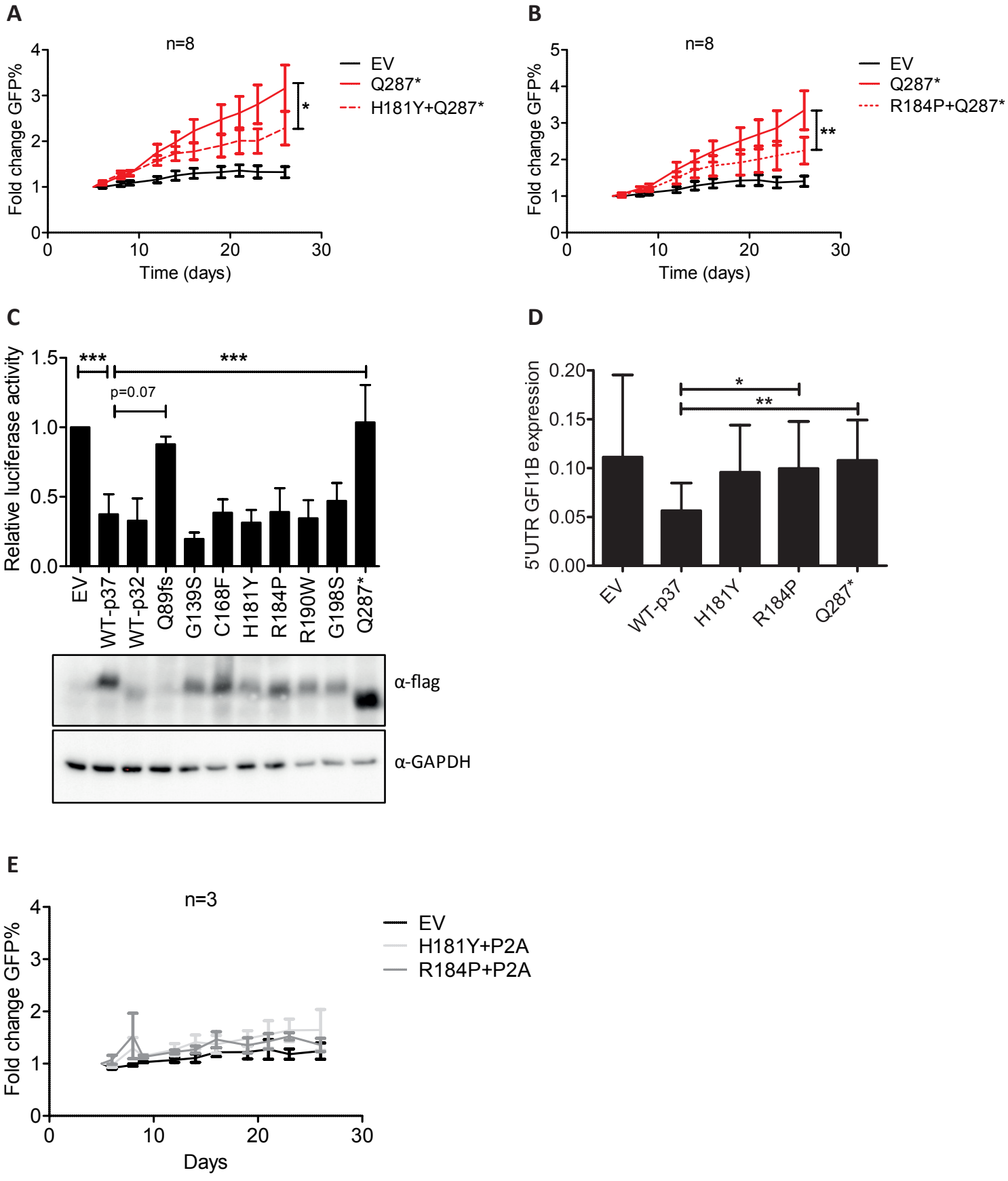
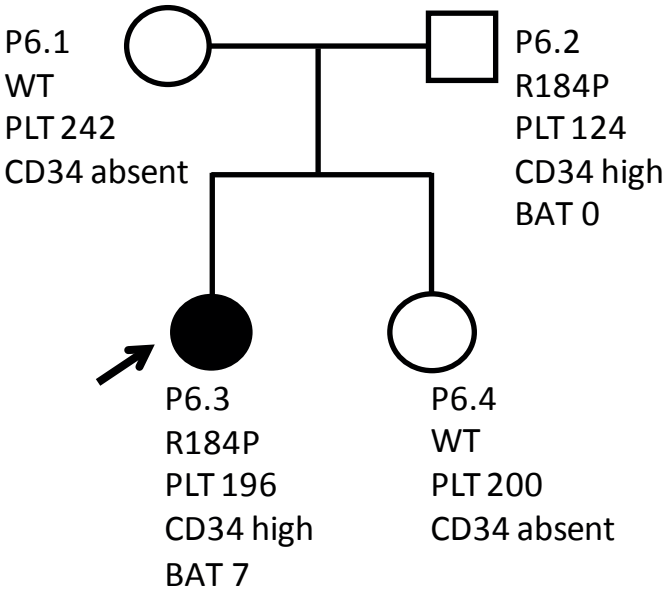
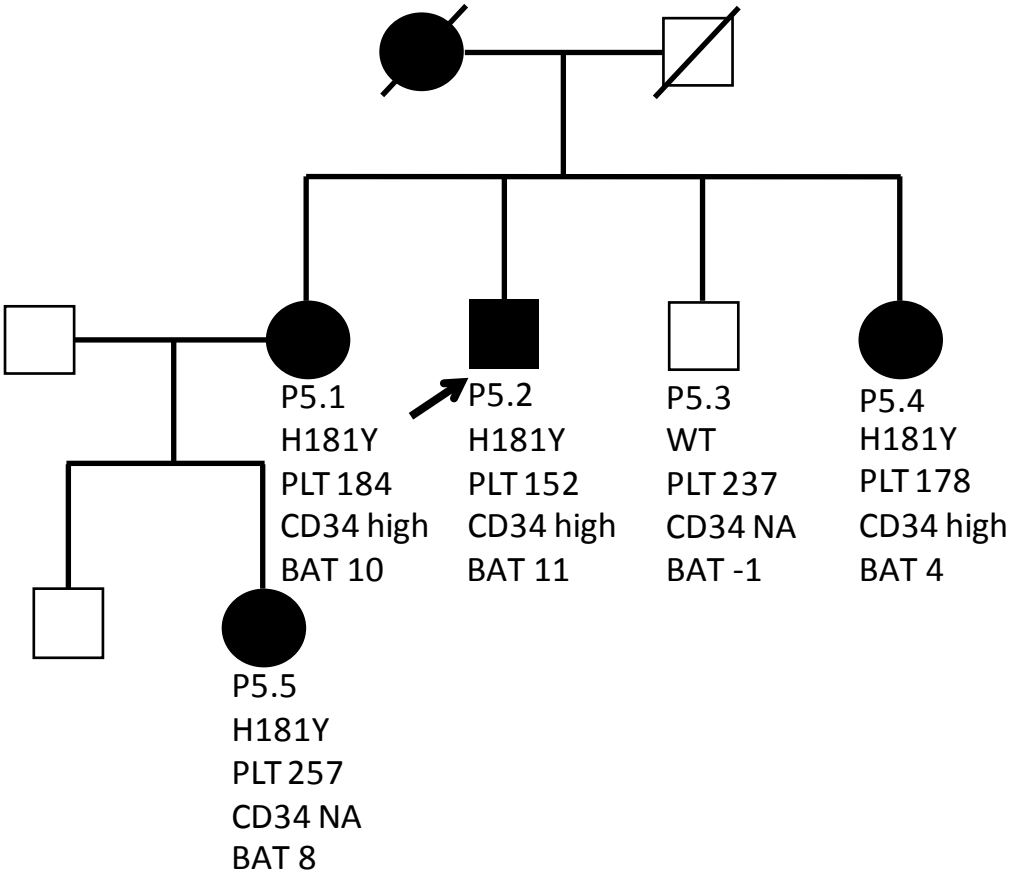

A
n=8
Fold change GFP%
Time (days)
EV
Q287*
H181Y+Q287*
*
B
n=8
Fold change GFP%
Time (days)
EV
Q287*
R184P+Q287*
**
C
Relative luciferase activity
***
***
p=0.07
EV
WT-p37
WT-p32
Q89fs
G139S
C168F
H181Y
R184P
R190W
G198S
Q287*
α-flag
α-GAPDH
D
5'UTR GFI1B expression
*
**
EV
WT-p37
H181Y
R184P
Q287*
E
n=3
Fold change GFP%
Days
EV
H181Y+P2A
R184P+P2A

Figure 3

A


P6.1
WT
PLT 242
CD34 absent
P6.2
R184P
PLT 124
CD34 high
BAT 0
P6.3
R184P
PLT 196
CD34 high
BAT 7
P6.4
WT
PLT 200
CD34 absent

B


P5.1
H181Y
PLT 184
CD34 high
BAT 10
P5.2
H181Y
PLT 152
CD34 high
BAT 11
P5.3
WT
PLT 237
CD34 NA
BAT -1
P5.4
H181Y
PLT 178
CD34 high
BAT 4
P5.5
H181Y
PLT 257
CD34 NA
BAT 8